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hereby certify that the annexed is a true copy of the Provisional specification in  
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UNIVERSITY OF SOUTH AUSTRALIA filed on 5 September 1997.

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**A U S T R A L I A**

**Patents Act 1990**

**PROVISIONAL SPECIFICATION**

for the invention entitled:

"A Method of Diagnosis"

The invention is described in the following statement:

- 1A -

## A METHOD OF DIAGNOSIS

The present invention relates generally to a method of diagnosing lung damage and more particularly, to a method of diagnosing alveolo-capillary membrane damage. The method of the present invention is useful *inter alia* for detecting lung damage such as that caused by noxious agents or as an undesirable side effect resulting from exposure to a therapeutic agent and for monitoring the progress of lung damage.

10 The bibliographic details of the publications referred to by author in the specification are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The gas/liquid interface of the lung is lined with a monomolecular layer comprising phospholipids, neutral lipids and specific proteins (surfactant proteins A, B, C and D, 20 herein referred to as SP-A, -B, -C and -D, respectively). Collectively known as "pulmonary surfactant", these compounds lower surface tension, decrease the work of breathing, and stabilise the lung by varying surface tension allowing alveoli of different sizes to co-exist.

25 Pulmonary surfactant phospholipids are synthesised by Alveolar Type II cells where they are stored in distinctive vesicles known as lamellar bodies. In response to a variety of stimuli, in particular physical distortion of the type II cells, the contents of the lamellar bodies are released into the hypophase, where they hydrate to form a 3-D lattice structure known as tubular myelin. The tubular myelin in turn supplies the monomolecular layer 30 at the gas/liquid interface that possesses the biophysical activity.

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The components of the monomolecular layer have a defined life and are constantly replaced. The disaturated phospholipids (DSP) are credited with reducing surface tension to the very low values thought to occur at low lung volumes, while cholesterol, the second most abundant pulmonary surfactant lipid, is thought to affect the rate of adsorption and  
5 the fluidity of newly released material. The system is extremely dynamic; in rats, dipalmitoylphosphatidylcholine, the main component of mammalian pulmonary surfactant, has a half-life of ~85 minutes in the alveolus with as much as 85% taken back into type II cells and reutilised (Nicholas *et al.*, 1990).

10 To date, four proteins, SP-A, -B, -C and -D have been shown to be uniquely associated with mammalian pulmonary surfactant. There is a general consensus that the extremely hydrophobic proteins (SP-B and -C) are functional components of the monomolecular layer, whereas the hydrophilic protein, SP-A appears to be more involved in pulmonary surfactant homeostasis and host defence, and SP-D is solely involved in host defence.

15

The adult respiratory distress syndrome (ARDS) represents a severe, diffuse lung injury caused by either direct, via the airways, or indirect, via the blood, trauma. The hallmark of ARDS is a deterioration in blood oxygenation and respiratory system compliance as a consequence of permeability edema. Whereas a variety of different insults may lead to  
20 ARDS, a common pathway probably results in the lung damage. Leukocyte activation within the lung, along with the release of oxygen free radicals, arachidonic acid metabolites, and inflammatory mediators such as interleukin-1, proteases, and tumor necrosis factor results in an increase in alveolo-capillary membrane permeability. With the loss of this macromolecular barrier, alveoli are flooded with serum proteins, which  
25 impair the function of pulmonary surfactant (Said *et al.*, 1965; Holm *et al.*, 1987). This creates hydrostatic forces that further exacerbates the condition (Jefferies *et al.*, 1988), leading to alveolar edema and a concomitant deterioration in gas exchange and lung compliance.

30

In the last decade, numerous methods for determining lung permeability have been assessed (Staub *et al.* 1990). Generally, these have relied upon detecting flux of radiolabels into, or out of, the lung. However, few have been applied clinically because of logistic problems with suitable scanners, stability, and specificity of the labels, and  
5 uncertainty over mathematical modelling (Staub *et al.* 1990). Further, lung damage, such as that induced by a noxious agent, has only been clinically detectable when sufficient damage has occurred for there to be changes in airway resistance or gas exchange. It is well accepted that this reflects relatively advanced lung disease.

10 Surfactant proteins are normally only found in appreciable amounts in the lung. In the airspaces, SP-A predominantly forms high molecular weight oligomers (~650 kDa) with Stokes radii of ~35 nm (Voss *et al.*, 1988). Although mature SP-B, which associates as a low  $M_r$  (~18 kDa) thiol dependent homo-dimer (Johansson *et al.*, 1991), is normally intimately associated with complexes of surfactant phospholipids, (Longo *et al.*, 1992),  
15 labelling studies in isolated type II cells suggest that at least some of the protein is secreted into the alveolus as hydrophilic, monomeric proprotein and processing intermediate with  $M_r$  of ~45 kDa and ~25 kDa, respectively (Weaver and Whitsett, 1989).

In work leading up to the present invention, the inventors have unexpectedly found that  
20 serum pulmonary surfactant levels provide an extremely sensitive diagnostic marker of lung damage, and in particular early stage lung damage.

Accordingly, one aspect of the present invention relates to a method of diagnosing lung damage in a mammal, said method comprising screening for the modulation of pulmonary  
25 surfactant levels in the body fluid of said mammal.

Reference to "body fluid" should be understood to include reference to fluids derived from the body of said mammal such as, but not limited to, blood (including all blood derived components, for example, serum and plasma), urine, tears, bronchial secretions or mucus  
30 and fluids which have been introduced into the body of said mammal and subsequently

removed such as, for example, the saline solution extracted from the lung following lung lavage. Preferably, the body fluid is blood or urine and even more preferably blood. Reference hereinafter to blood should be read as including reference to all other body fluids.

5

The term "mammal" as used herein includes humans, primates, livestock animals (e.g. horses, cattle, sheep, pigs, donkeys), laboratory test animals (e.g. mice, rats, rabbits, guinea pigs), companion animals (e.g. dogs, cats) and captive wild animals (e.g. kangaroos, deer, foxes). Preferably, the mammal is a human or a laboratory test animal.

10 Even more preferably, the mammal is a human.

The term "lung damage" encompasses, but is not limited to, lung damage due to, for example, a congenital abnormality or an acquired abnormality such as that due to the onset of an autoimmune condition, changes in pressure/volume relationships in the lung, exposure of said mammal to a foreign agent (for example cigarette smoke or dust), a noxious or toxic agent (for example solvents or fumes) or is an undesirable side effect resulting from exposure to a therapeutic agent. Said damage may be, for example, characterised by alveolo-capillary membrane damage.

20 Reference hereinafter to "pulmonary surfactant" should be read as including reference to all forms of pulmonary surfactant and derivatives thereof including but not limited to pulmonary phospholipids, pulmonary neutral lipids and pulmonary surfactant proteins, and includes all subunit molecules including, by way of example, the precursor, proprotein and intermediate forms of SP-B. Examples of pulmonary surfactant proteins include SP-A, -  
25 B, - C and -D. Preferably, said pulmonary surfactant is SP-A, -B, -C or -D.

Accordingly, there is provided a method of diagnosing lung damage in a mammal, said method comprising screening for the modulation of one or more of SP-A, -B, -C or -D levels in the blood of said mammal.

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- 5 -

Levels of circulating SP-A and SP-B depend not only on the relative sizes of the proteins and lung permeability but also the form available to breach the membrane barriers. SP-A binds phospholipid avidly to the extent that there is little of it free in alveoli fluid. In contrast, the predominant form of alveolar immunoreactive SP-B, proprotein and  
5 processing intermediate are not bound to surface lipids, possibly allowing freer entry into circulation. Further, that the plasma SP-B/SP-A ratio varies with lung function suggests that plasma SP-B is a more sensitive marker of changes in lung permeability than is SP-A.

In a most preferred embodiment, the present invention relates to a method of diagnosing  
10 lung damage in a mammal said method comprising screening for the modulation of SP-B levels in the blood of said mammal.

In a particular aspect, said lung damage may be alveolo-capillary membrane damage.

15 "Derivatives" of said surfactants includes fragments, parts, portions, mutants and analogs thereof. Derivatives may be derived from insertion, deletion or substitution of an amino acid. Amino acid insertional derivatives include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced  
20 into a site in the protein. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place.

25 The method of the present invention is particularly useful in detecting early stage lung damage. "Early stage" is defined as the period during which the onset and development of lung damage is undetectable or else cannot be confirmed without the aid of one or more invasive procedures. For example, the method of this invention has application in detecting early changes in lung permeability in smokers. "Early stage" should also be  
30 understood to include low levels of lung damage such as for example, mild but chronic

lung damage. Early changes in lung permeability which may be associated with neutrophil recruitment and initial destruction of lung connective tissue by elastase and reactive oxygen species may be marked by an increase in plasma SP-B levels despite the absence outwardly of any visible symptoms of lung damage.

5

Accordingly, there is provided a method of diagnosing early stage lung damage in a mammal, said method comprising screening for the modulation of pulmonary surfactant levels in the blood of said mammal.

10 Preferably said pulmonary surfactant is SP-A, -B, -C or -D and even more preferably SP-B.

In a most preferred embodiment there is provided a method of detecting early stage lung damage in a mammal, said method comprising screening for the modulation of SP-B levels  
15 in the blood of said mammal.

In particular, said lung damage may be alveolo-capillary membrane damage.

Although not intending to limit the invention to any one theory or mode of action, alveolo-  
20 capillary membrane damage causes an increase in alveolo-capillary permeability. Although immunoreactive SP-A and SP-B are not normally present in appreciable amounts in the systemic circulation, it is thought that the appearance of additional pulmonary surfactant proteins in the serum of patients with lung damage occurs as the result of changes in alveolo-capillary permeability.

25

Accordingly, the term "modulation" refers to increases and decreases in serum pulmonary surfactant levels. Preferably, said modulation is an increase in blood pulmonary surfactant levels.

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According to this preferred embodiment there is provided a method of diagnosing lung damage in a mammal said method comprising screening for an increase in pulmonary surfactant levels in the blood of said mammal.

- 5 Preferably, said pulmonary surfactant is SP-A, -B, -C or -D and even more preferably SP-B.

In particular, the lung damage may be early stage lung damage and most particularly alveolo-capillary membrane damage.

10

According to this most preferred embodiment, there is provided a method of diagnosing early stage alveolo-capillary membrane damage in a mammal, said method comprising screening for an increase in SP-B levels in the blood of said mammal.

- 15 Although the preferred method is to detect an increase in blood pulmonary surfactant levels, the detection of a decrease in said surfactant levels may be desired under certain circumstances. For example, to monitor improvement in alveolo-capillary membrane morphology during the course of therapeutic treatment of patients presenting with alveolo-capillary membrane damage.

20

Accordingly, another aspect of the present invention provides a method of monitoring changes in the extent of lung damage in a mammal said method comprising screening for the modulation of pulmonary surfactant levels in the blood of said mammal.

- 25 Preferably, said pulmonary surfactant is SP-A, -B, -C or -D and even more preferably SP-B.

In particular, the lung damage may be alveolo-capillary membrane damage.

30

In a most preferred embodiment there is provided a method of monitoring for an increase in the extent of alveolo-capillary membrane damage in a mammal said method comprising screening for an increase in SP-B levels in the blood of said mammal.

- 5 In yet another most preferred embodiment there is provided a method of monitoring for a decrease in the extent of alveolo-capillary membrane damage in a mammal said method comprising screening for a decrease in SP-B levels in the serum of said mammal.

The method of the present invention has widespread applications, including but not limited  
10 to, as a non-invasive clinical monitor of lung function and the onset of alveolo-capillary membrane damage of individuals exposed to foreign agent or a noxious or toxic agent such as individuals who smoke or individuals who are involved in occupations such as welding, spray painting, fibreglass manufacture or involving exposure to passive smoking, which may potentially result in lung damage. The method of the present invention also has  
15 application in assessment of the lung health status of any individual irrespective of any perceived predisposition or possibility of having acquired a degree of lung damage.

The method of the present invention extends to diagnosing the severity of lung damage in a mammal based upon an analysis of quantitated pulmonary surfactant levels in the blood  
20 of said mammal. Said pulmonary surfactants may be quantitated and analysed either separately or relative to one another. For example, lung injury results in a differential change in blood SP-A and SP-B such that the ratio of SP-B/SP-A is inversely related to lung function.

- 25 Screening of pulmonary surfactant levels in serum of a mammal can be achieved via a number of techniques such as functional tests, enzymatic tests or immunological tests. Functional tests may include detecting SP-A or -B by their ability to affect release or re-uptake of surfactant or by detecting host defence properties. SP-C may be detected by measuring associated palmitates. Immunological tests may include contacting a serum  
30 sample with an antibody specific for a pulmonary surfactant (or group of pulmonary

surfactants) or its derivatives thereof for a time and under conditions sufficient for an antibody-pulmonary surfactant complex to form, and then detecting said complex.

In one particular preferred method the target surfactant molecules in the serum sample are  
5 exposed to a specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with an antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The  
10 complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most  
15 commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however,  
20 a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes  
25 include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-  
30 antibody. The substrate will react with the enzyme linked to the second antibody, giving a

qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically  
5 coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-  
10 hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or  
15 bioluminescent molecules, may also be employed.

Another aspect of the present invention provides a diagnostic kit for assaying serum samples comprising in compartmental form a first compartment adapted to contain an agent for detecting pulmonary surfactant and a second compartment adapted to contain reagents  
20 useful for facilitating the detection by the agent in the first compartment. Further compartments may also be included, for example, to receive a biological sample. The agent may be an antibody or other suitable detecting molecule.

Further features of the present invention are more fully described in the following  
25 Examples. It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention. It should not be understood in any way as a restriction on the broad description of the invention as set out above.

### EXAMPLE 1

#### Determination of Circulating Surfactant Protein-A and -B Levels in Smokers and Non-smokers

5 Since cigarette smoking may acutely and reversibly increase lung epithelial permeability through smoke mediated release of vasoactive neuropeptides (tachykinins) from sensory nerves in the airways (Germonpre *et al*, 1995; Geppetti *et al*, 1993; Lei *et al*, 1993; Nadel & Borson, 1991), smokers are requested to refrain from smoking for at least 4 h prior to screening. Two ml of peripheral blood is drawn from an antecubital vein and centrifuged  
10 in lithium heparin tubes at 5,000 rpm for 5 min at room temperature (Megafuge; Heracus-Christ; Osterode, Germany) immediately following collection.

In order to free the immuno-reactive-SP-A and -B from any associated plasma or surfactant components, aliquots are treated with EDTA, SDS, and Triton X-100. Briefly, 125µl of  
15 each plasma sample is diluted in 500µl of 10 mM Tris, 1 mM EDTA containing 0.25 % BSA (pH 7.4). After vortexing at room temperature for 10 min, 125µl of solution containing 3% SDS and 12% Triton X-100 (vol/vol) is added to each and the samples again vortexed for 10 min.

20 SP-A and -B are determined by ELISA inhibition assays (Doyle *et al*, 1994; Yogalingam *et al*, 1996) using Po-A and -B, antibodies raised against alveolar proteinosis SP-A and mature SP-B, respectively. Po-B reacts with mature SP-B, the processing intermediate, and SP-B proprotein.

25 Briefly, serial dilutions of the samples in 136.8 mM sodium chloride, 8.1 mM disodium hydrogen phosphate, 2.6 mM potassium chloride, 0.7 mM potassium dihydrogen phosphate containing 0.02% sodium azide, 0.05% v/v Tween 20 and 0.25% BSA (wt/vol) are incubated in an ELISA plate (#2595; Costar, Cambridge, MA) with aliquots of either Po-A or -B. Free antibody is captured using a second ELISA plate coated with purified  
30 SP-A or -B (1µg/ml), and the amount quantified using alkaline phosphatase conjugated IgG

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against rabbit immunoglobulins and 15 mM disodium p-nitrophenyl phosphate in 1.0 M  
dichanolamine and 0.5 mM magnesium chloride as a substrate. After ~1 h the absorbance  
of the substrate is measured at 405 nm using a Dynatech MR 5000 reader (Dynatech  
Laboratories, Chantilly, VA). An AssayZap program (Biosoft, Ferguson, MO) is used to  
5 generate a standard curve and to compute the concentration of SP-B in each sample. All  
samples are assayed in duplicate at 4 serial dilutions. Standards, assayed in quadruplicate,  
are included in each ELISA plate at 8 serial dilutions (ranging from, SP-A: 1.95 to 250  
ng/ml; SP-B: 7.8 to 1000 ng/ml;  $r^2 > 0.99$  for both).

10

## EXAMPLE 2

### SP-A and SP-B plasma levels in smokers

In healthy subjects with no history of cardiorespiratory disease, whereas plasma SP-A  
levels are similar in smokers ( $165 \pm 7$  ng/ml; mean  $\pm$  SE;  $n=10$ ) and non-smokers ( $191$   
15  $\pm 10$  ng/ml;  $n=10$ ), plasma SP-B levels are ~2-fold higher in the smokers (smokers:  
 $3,480 \pm 640$  ng/ml; non-smokers:  $1,687 \pm 143$  ng/ml). Indeed, there is no overlap  
between the plasma SP-B levels between the two groups, which are determined in a blind  
randomized manner (Fig. 1).

20

## EXAMPLE 3

### Public Health detection of previously un-detectable lung damage in smokers

Cigarette smoke inhalation damages the airway and lung parenchyma with a consequent  
increase in alveolo-capillary permeability. Smoke induced lung damage is currently only  
25 detectable when sufficient damage has occurred for there to be changes in airway resistance  
or gas exchange. This reflects relatively advanced lung disease. Elevated levels of  
surfactant proteins in the blood or blood product of smokers is used to detect both active  
and passive smoke induced lung damage. This data is compared with non-smoking levels  
from established epidemiological data.

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#### **EXAMPLE 4**

##### **Monitoring of therapeutic, lung toxic, drugs - for example methotrexate**

Methotrexate is a commonly used immuno suppressive drug for the treatment of a variety  
5 of conditions including rheumatoid arthritis. However, a side effect of methotrexate is  
lung damage which has, to date, been detected by symptoms such as changes in blood  
gases or sophisticated lung function tests. Said methods detect advanced lung damage.  
Blood or blood product surfactant protein levels are used to monitor the safety of  
methotrexate therapy by detecting any increase in alveolo-capillary permeability.  
10 Monitoring comprises a preliminary test followed by intermittent (daily, weekly or  
monthly) testing.

#### **EXAMPLE 5**

##### **Monitoring of radiotherapy induced lung damage**

15

Radiotherapy which targets structures in the chest may cause lung damage which can lead  
to acute respiratory failure. Blood or blood product surfactant protein levels are used to  
monitor the safety of radiotherapy, which is usually a course of treatment, and to reduce  
its dose or frequency if increased alveolo-capillary permeability is detected. Where no  
20 evidence of lung damage is detected, greater doses of radiotherapy can be prescribed.

#### **EXAMPLE 6**

##### **Monitoring lung damage in race horses**

25 During strenuous exercise, pulmonary vascular pressures are elevated and result in  
increased lung water and increased alveolo-capillary permeability in race horses. This  
results in rupture of pulmonary blood vessels which manifest as alveolo blood. Blood or  
blood product surfactant protein levels are monitored during the training, racing of race  
horses and during recovery from exercise induced lung damage.

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by its Patent Attorneys  
DAVIES COLLISON CAVE

FIGURE 1

Serum Surfactant Proteins-A and -B

